Original Research Article

Antifungal Activity of Palmatine against Strains of *Candida* spp. Resistant to Azoles in Planktonic Cells and Biofilm

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A B S T R A C T

The incidence of fungal infections has increased and it is necessary to seek new antifungals that also act against resistant strains. Palmatine is a protoberberine alkaloid noteworthy for its various pharmacological properties, including antifungal properties. The objective of this study was to evaluate the activity of palmatine against *Candida* spp. strains resistant to azole in planktonic cells and biofilm, and to elucidate the possible mechanism of cell death. Palmatine showed an antifungal activity with MICs ranging from 32 to 128 μg/mL. In the formed biofilms, palmatine reduced by 50% cell viability at the concentration of 20x MIC (*C. albicans*) and 10x MIC (*C. tropicalis* and *C. parapsilosis*). In the formed biofilms, the same percentage of inhibition was achieved at a concentration of 1x MIC (*C. albicans*) and 2x MIC (*C. tropicalis* and *C. parapsilosis*). It was found that palmatine causes damage to DNA, promotes mitochondrial depolarization and increasing the externalization of phosphatidylserine, suggesting cell death by apoptosis. The antifungal action of palmatine in *Candida* spp. resistant to azoles in planktonic cells and biofilm formed and in formation, as well as the fact of not having presented cytotoxicity in mammalian cells, point to a promising new anti-candida molecule.

Introduction

Species of the genus *Candida* occupy the fourth place among the most common microorganisms isolated in hospital sepsis (Kumar *et al.,* 2015). Fungal infections may progress rapidly, leading to an increase in mortality rates and hospital costs due to an increased hospitalization time (Kazak *et al.,* 2014). The incidence of these infections has been increasing over the years due to the increasing number of immunocompromised patients, increased invasive medical procedures and the use of broad spectrum antibiotics (Yapar *et al.,* 2014).

It is estimated that in 65-80% of infections, microorganisms are organized in the form of biofilms (Valentin *et al.,* 2012; Bonhomme and d'Enfert, 2013). Biofilms are communities of microorganisms organized within an extracellular matrix allowing them to live in harsh environments, as well as escaping the host's immune defense system, representing a major virulence factor (Yu *et al.,* 2012). *Candida* spp. species have the ability to form biofilms both in the host tissue and in medical devices (Zavrel and White, 2015; Tabbene *et al.,* 2016).

Due to the increase in fungal infections, low number of available antifungal drugs and increased antifungal resistance, it is necessary to seek new therapeutic strategies (Guo *et al.,* 2008). In this context, palmatine is a protoberberine alkaloid noteworthy for its various biochemical and pharmacological properties, including antifungal properties (Mi *et al.,* 2015; Dumont and Monari, 2015). *Radix berberidis*, *Coptidis rhizoma* and *Cortex phellodendri* are commonly used as Chinese traditional medicines containing isoquinolínico alkaloids, and they have been applied for more than 2,000 years because of the antimicrobial effects (QI *et al.,* 2013). A previous study conducted by our research group demonstrated an interesting activity of berberine against strains of *Candida* spp. and *Cryptococcus neoformans* (Da Silva *et al.,* 2016). The aim of the present study was to evaluate the antifungal activity of palmatine against different azole-resistant *Candida* strains in planktonic cells and biofilm. The mechanisms of action were assessed by flow cytometry and comet assay (standard single cell gel electrophoresis).

Materials and Methods

Yeast strains

Twenty strains of azoles-resistant *Candida* spp. (6 *C. albicans*, 7 *C. tropicalis*, 6 *C. parapsilosis, 1 C. glabrata)* were used. These strains are from the yeast collection of the Laboratory of Bioprospection and Experiments in Yeast (LABEL/FF/UFC). The strains were incubated on Sabouraud dextrose agar (Himedia, Mumbai, India) at 37°C for 24 h, and were then cultivated on CHROMagar Candida® medium (Himedia) to assess their purity.

Cultivation and L929 cell proliferation inhibition using the MTT Test

The procedure was performed according to Da Silva *et al.,* (2016).

In vitro antifungal activity

The broth microdilution (BMD) antifungal susceptibility test was performed according to the document M27-S4 (Clinical and Laboratory Standards Institute, 2012) (CLSI) (CLSI, 2012) using RPMI-1640 broth (pH 7.0) buffered with 0.165 M MOPS [3-(N-morpholino) propanesulfonic acid] (Sigma Chemical Co. St Louis, MO, USA). Palmatine (Sigma Chemical) was dissolved in dimethyl sulfoxide (DMSO; Sigma Chemical) and tested at concentrations ranging from 0.5 to

256 μg/mL. The yeasts and compound were incubated in 96-well culture plates at 35 °C for 24 h and the results were examined visually, as recommended by CLSI. The minimum inhibitory concentration (MIC) of compound was determined as the concentration that inhibited 50% of fungal growth. The strains *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used as controls (CLSI, 2012).

Yeasts suspensions preparation

Cell suspensions were prepared from cultures in the exponential growth phase. The cells were harvested by centrifugation (1600 *g* for 10 min at 4 °C), washed twice with 0.85% saline solution (1200 *g* for 5 min at 4 °C) and then resuspended ($\sim10^6$ cells/mL) in HEPES buffer (pH 7.2) supplemented with 2% glucose. Ampho B was used as a cell death control (Da Silva *et al.,* 2014).

Mechanism of action studies

To determine their cell density, mitochondrial transmembrane potential, reactive oxygen species, annexin V staining and DNA damage (presence and absence of enzyme FPG), *C. albicans* azoles-resistant strain was exposed to various concentrations (MIC, 2x MIC and 4x MIC; see Table 1) of palmatine. Treatment with amphotericin B (Ampho B; 4 μg/mL) (Sigma Chemical) was used as a positive control for cell death. All of the experiments were performed in triplicate in three independent experiments to according previous studies already published by our research group (Da Silva *et al.,* 2014; Da Silva *et al.,* 2016).

Biofilm viability

The procedure was performed according to Da Silva *et al.,* (2016).

Inhibition of biofilm formation

The inhibition of biofilm formation followed the methodology described above with small modifications. For this test, one strain of each *Candida* species (see Table 1) incubated in yeast extract-peptone-dextrose at 35 °C for 20 to 24 h was used. The palmatine (8 to 1280 μg/mL) was added at the time inoculation. After 24 h the test product was discarded, and the wells were washed twice with PBS. Measurement of the metabolic activity of the biofilm cells was evaluated using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; 1 mg/ml) colorimetric assay. Reading of the results was conducted in a microplate reader at 540 nm (Pires *et al.,* 2013).

Statistical analysis

In vitro susceptibility experiments were repeated at least three times on different days and geometric means were used to compare the MIC values. The data obtained from the flow cytometry and alkaline comet assays were compared using a one-way analysis of variance (ANOVA) followed by the Newman-Keuls test ($p<0.05$). Mean absorbance values from the biofilm formation assay were compared using one-way ANOVA followed by the Tukey test (p <0, 05).

Results and Discussion

Citotoxic activity in murine fibroblasts

After 72 h exposure to palmatine no showed cytotoxicity against murine fibroblasts (L929 strain), because didn't interfered with the proliferation of cells as well as untreated control cells (p <0.05). The median inhibitory concentration (IC50), lower concentration of an agent which inhibits 50% of cell growth study was higher than the range tested, 100 μg / mL.

Antifungal effects of palmatine against azoles-resistant *Candida* spp. strains

Palmatine were able to inhibit the growth of all azoles-resistant strains used in the present work (Table 1). The MIC values ranged from 32 – 128 μg/ mL. Based on these findings, experiments were devised aiming to elucidate the mechanisms involved in the antifungal action of the palmatine against *C. albicans* (Table 1).

Loss of cell viability in azoles-resistant *Candida* spp. after treatment with palmatine

As shown in Figure 1, when the azoles-resistant cells were treated with palmatine for 3, 6, 12 and 24 h they exhibited significant decreases in cell viability (P<0.05).

Increased intracellular levels of ROS in azoles-resistant *Candida* spp. after exposure to palmatine

As shown in Figure 2, cells of *C. albicans* resistant to azoles when treated with palmatine showed no significant differences in the levels of intracellular ROS compared with the negative control.

Yeast Δψm changes are induced by palmatine

Significant (p<0.05) changes in the mitochondrial transmembrane potential were observed when the yeast cells were exposed to increasing concentrations of the palmatine in comparison to untreated cells (Figure 3).

Externalization of phosphatidylserine in yeast cells

In Figure 4, *Candida albicans* treated with palmatine showed significant increases (P<0.05) in the percentage of apoptotic cells when compared to the control group.

DNA damage

As shown in Figure 5, palmatine induced a significant increases (P<0.05) in DNA damage in the azoles-resistant *Candida* spp. in comparison to untreated cells. Therefore, the azoles-resistant strains that had been incubated with FPG no showed a difference significant in the damage index values after the treatment with palmatine in comparison to the control group (P <0.05).

Effect of palmatine on the formed biofilm

As shown in Figure 6, *C. albicans* in the concentration of 20x MIC (1280 μg/ mL) caused a reduction close to 50% cell viability of the biofilm growth compared to the control. However, *C. tropicalis* demonstrated the same reduction with 10x MIC (640 μg/ mL) and *C. parapsilosis* with 10x MIC (320 μg/ mL).

In relation to inhibition of biofilm formation, as shown in Figure 7, *C. albicans* in the concentration of MIC (64 μg/ mL) caused a reduction close to 50% cell viability of the biofilm compared to the control. However, *C. tropicalis* demonstrated the same reduction with 2x MIC (128 μg/ mL) and *C. parapsilosis* with 2x MIC (64 μg/ mL).

The results of this work show that palmatine has an activity in different *Candida* species, such as *C. albicans*, *C. tropicalis*, *C. parapsilosis* and *C. glabrata*. The MICs found ranged from 32 to 128 μg/mL. The lowest MIC value was for *C. parapsilosis* strains. Our results are in agreement with the work conducted by (Park *et al.,* 1999) with sensitive clinical strains, which identified a higher antifungal activity of palmatine against *C. parapsilosis* strains, showing a MIC lower than 16 μg/mL. In *C. albicans*, *C. glabrata*, *C. guilliermondii* and *C. krusei* strains, MICs were higher than 500 μg/mL. The emergence of drugs that affect strains resistant to azoles is extremely important since these drugs are

chosen by most Brazilian hospitals (Storti *et al.,* 2012). After the antifungal action of palmatine against *Candida* spp. strains was proven, a cytotoxicity assay was performed in L929 cells. Palmatine showed no cytotoxicity regarding the cells under study, including at the highest concentration used, i.e., 100 µg/mL. The absence of cytotoxic activity in mammalian cells reinforces the importance of studying palmatine as a potential antifungal agent.

Upon assessing cell density, it was observed that *C. albicans* cells, when exposed to palmatine at the concentration 4x MIC, showed a significant decrease in cell density (p<0.05). According to Figure 1, it can be observed that palmatine at the MIC concentration appears to demonstrate a cytostatic action, since the number of viable cells did not have significant increases during the study period. Da Silva *et al.,* (2016) also found a decrease in the number of viable cells when strains of *Candida* spp. and *Cryptococcus neoformans* were treated with berberine at the concentration 4x MIC. This decrease was also greater than the death in the control.

Mitochondria are essential for the survival of eukaryotic cells and, at the same time, these organelles have the necessary structures for cell suicide, meaning that they are capable of controlling cell death (Galluzzi *et al.,* 2010). In this work, the mitochondrial function in *Candida albicans* cells was affected after exposure to palmatine. Changes to the mitochondrial membrane potential (Δψm) may be caused by the opening of pores. Such pores allow releasing protons and pro-apoptotic factors into the cytosol, such as cytochrome c (Hwang *et al.,* 2014; Hwang *et al.,* 2012; Da Silva *et al.,* 2014). This mitochondrial membrane permeability is an irreversible point in the cascade of events leading to cell death by the intrinsic apoptosis pathway (Galluzzi *et al.,* 2010).

The mitochondrial depolarization process was assessed using the fluorescent nucleophilic marker rhodamine 123. It is sequestered inside mitochondria when the membrane potential is unchanged. Thus, viable cells present a higher fluorescence due to a greater quantity of rhodamine 123 bonded to its internal positive charges. Depolarized mitochondria, however, have a lower affinity to staining, emitting a lower fluorescence (Da Silva *et al.,* 2014). *C. albicans* cells treated with palmatine showed high rates of depolarized cells at the three tested concentrations (MIC, 2x MIC and 4x MIC), evidencing that palmatine damages the mitochondrial membrane.

Mitochondrial dysfunction and the increase in intracellular production of reactive oxygen species (ROS) play an important role in inducing apoptosis. ROS such as peroxide oxygen, superoxide radicals and hydroxyl radicals, when accumulated excessively in the cell, cause molecular damage leading to cell death. The imbalance between excessive ROS rate and low intracellular antioxidant defenses leads to oxidative stress, which is the major cause of apoptosis (Choi and Lee, 2015; Hwang *et al.,* 2012).

Palmatine, at the tested concentrations, did not affect the production of reactive oxygen species (ROS) in relation to the control. It can also be noted that at the concentrations MIC, 2x MIC and 4x MIC, at a 12-24 h period, there was a significant decrease in ROS in relation to the control, probably due to the antioxidant action of palmatine.

A study conducted by (Pustovidko *et al.,* 2013) demonstrated that palmatine and berberine can accumulate in isolated mitochondria or in mitochondria of live cells, and effectively protect them from oxidative damage. Ali and Dixit (2013) conducted a study in which palmatine was used to treat skin cancer in mice. These researchers found

that palmatine promoted a decrease in lipid peroxidation levels, increasing the levels of enzymes involved in the antioxidant process such as catalase, superoxide dismutase and glutathione (Ali and Dixit, 2013).

Fig.1 Density cell after treatment with palmatine
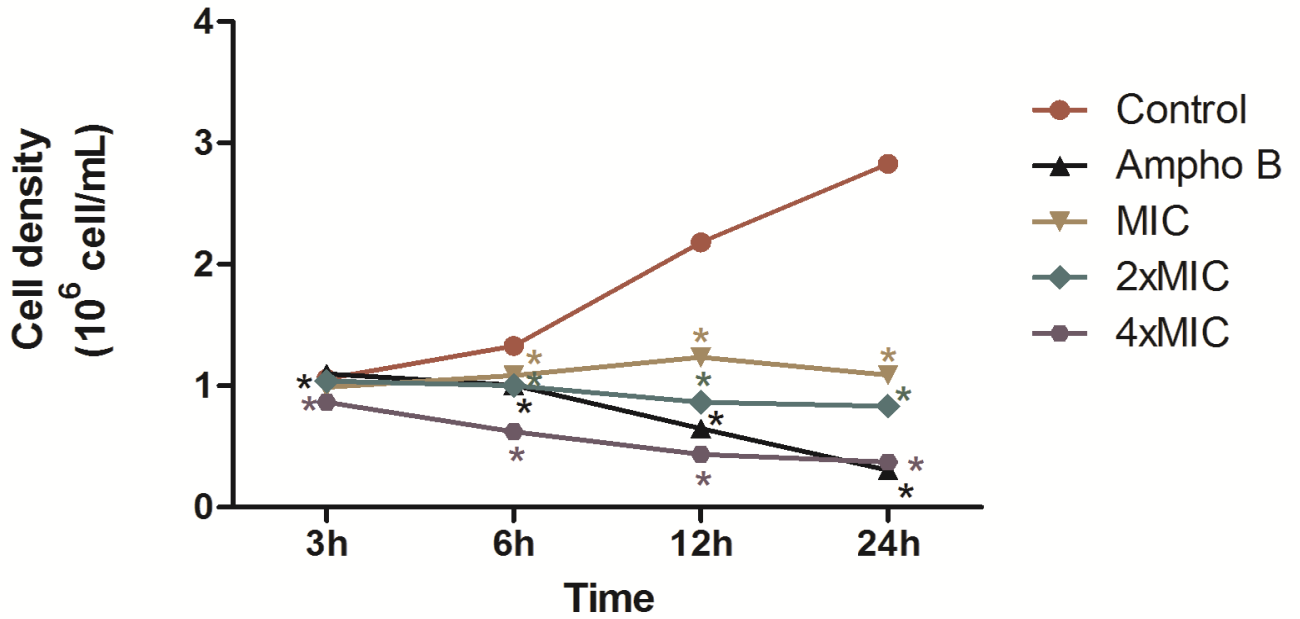
Fig.2 ROS formation after treatment with palmatine
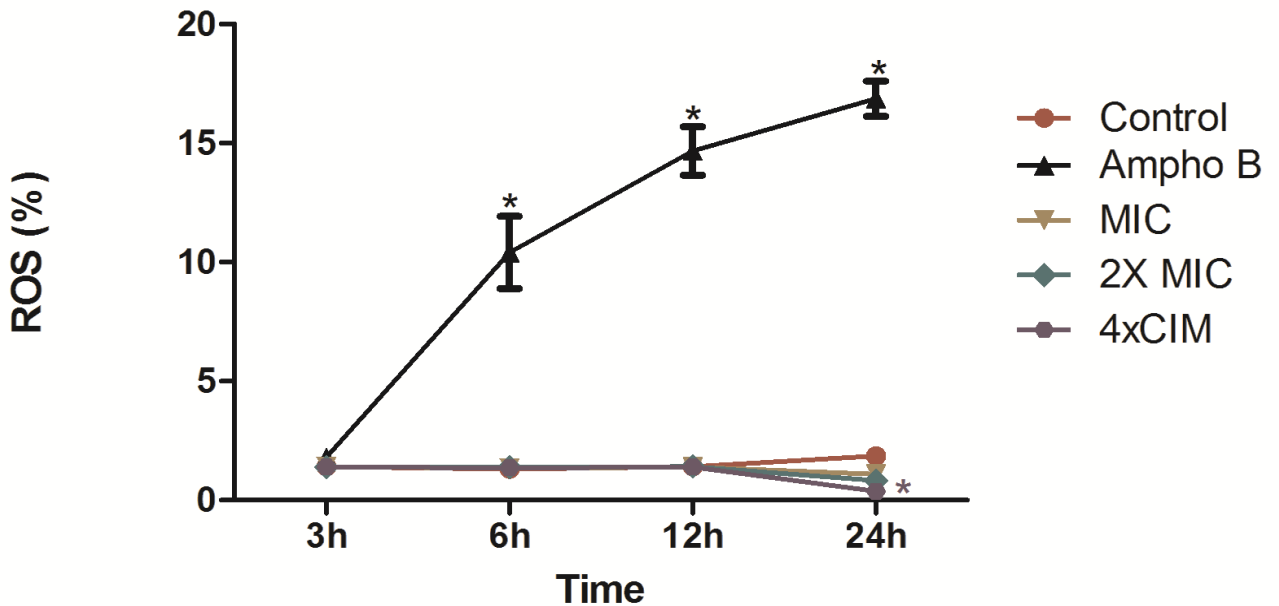
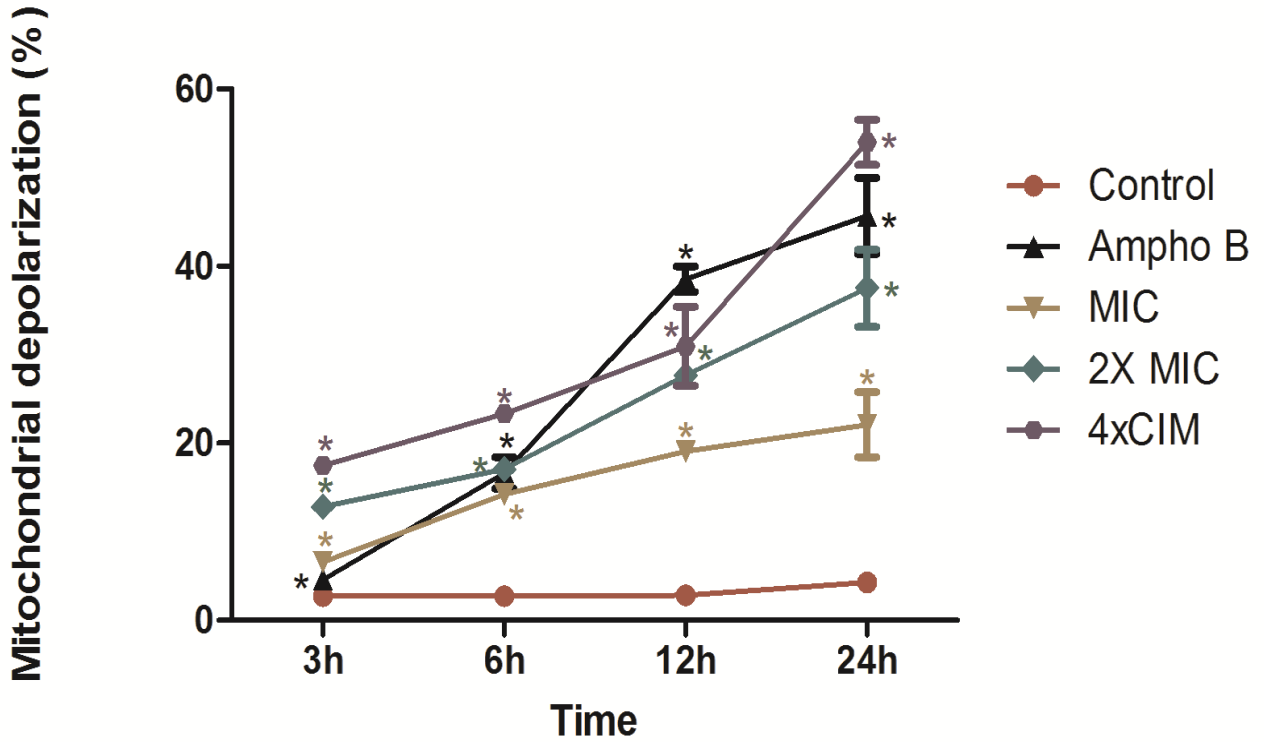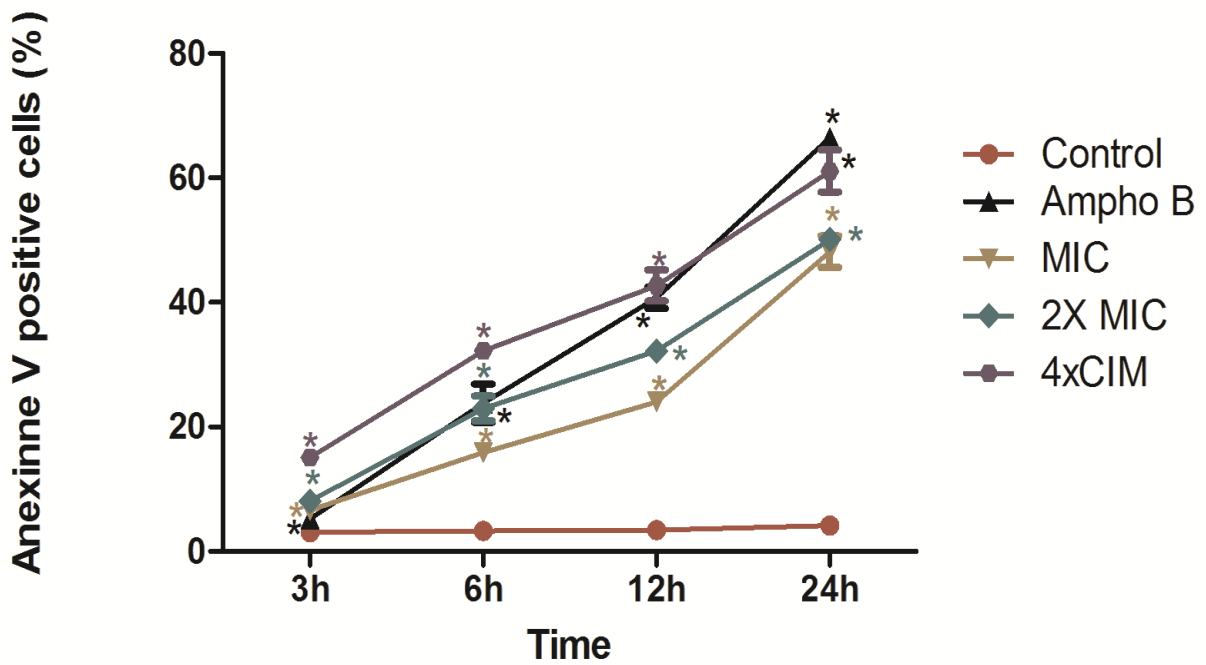**Fig.3** Effect of palmatine on the mitochondrial transmembrane potential
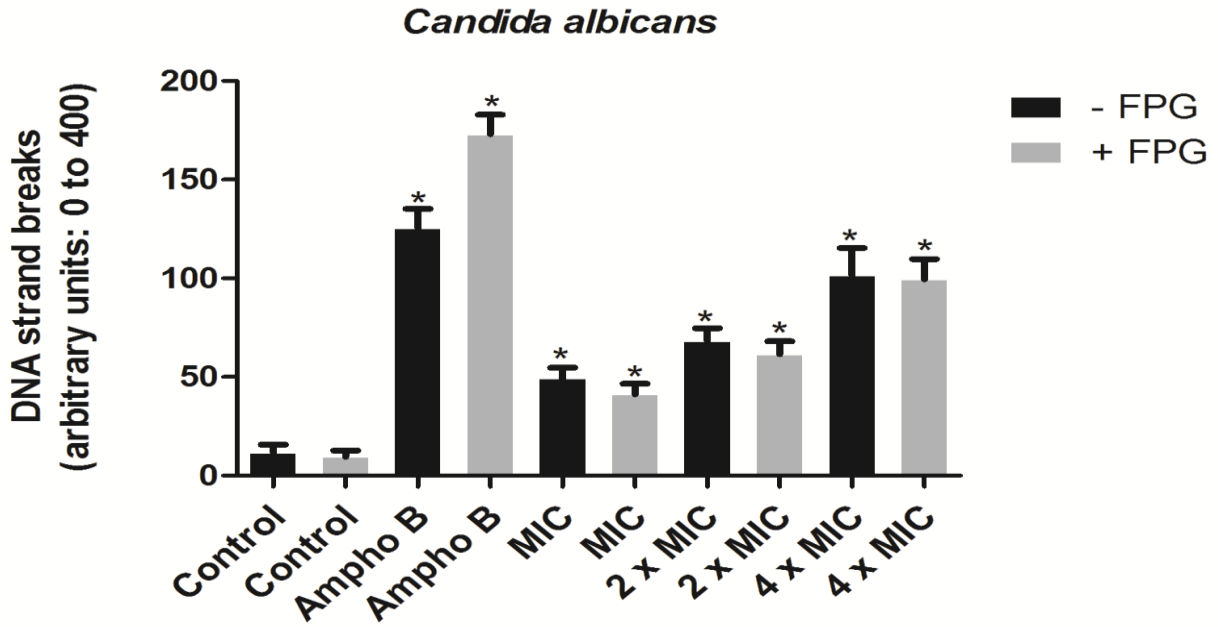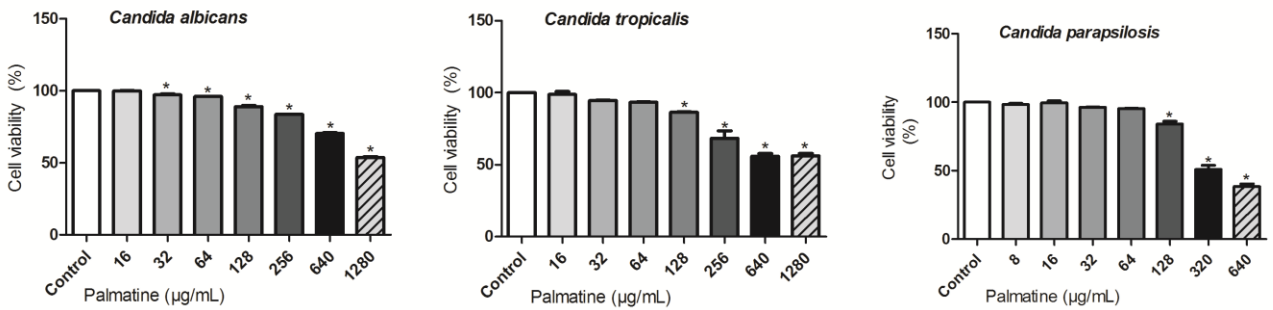

Fig.4 Effect of palmatine on phosphatidylserine externalization
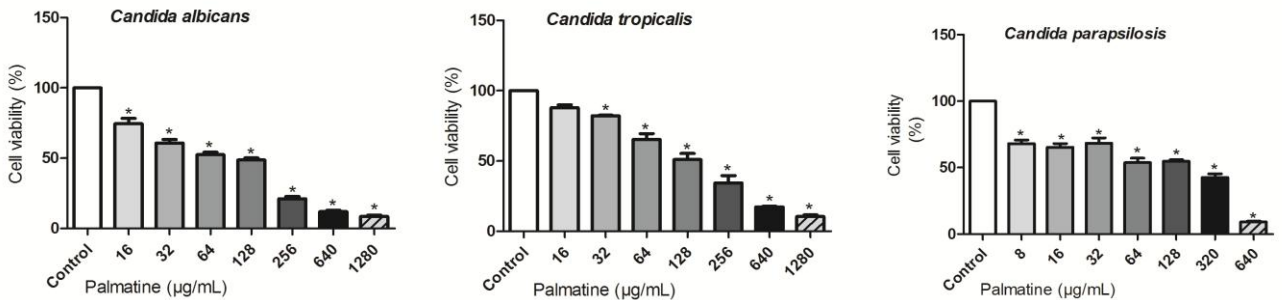
Fig.5 Effect of palmatine on the DNA damage index and on the distribution of oxidative DNA damage

Fig.6 Effect of palmatine on the metabolic activity of biofilms formed

Fig.7 Effect of palmatine on inhibition of biofilm formation

Table.1 Effect of palmatine against azole-resistant strains of *Candida* spp.

Strain [a]			MIC Values of MIC[b] (µg/mL)	
	Origin	Nº Genbank	Resistance Phenotype	Palmatine
C. albicans 1	Blood	KJ740179	FLC, ITRA, VRZ	64
C. albicans 2	Blood	KJ740175	FLC, VRZ	64
C. albicans 3/***	Blood	AB861478	FLC, ITRA	64
C. albicans 4	Blood	KJ740176	FLC, ITRA, VRZ	64
C. albicans 5	Blood	KJ740174	FLC, ITRA, VRZ	64
C. albicans 6	Blood	AB861479	FLC, VRZ	128
C.parapsilosis 1	Blood	KJ740188	FLC, ITRA	32
C. parapsilosis 2	Blood	AB861485	FLC, ITRA	64
C.parapsilosis 3	Blood	KJ740191	FLC, ITRA	32
*C. parapsilosis 4***	Blood	KJ740186	FLC, ITRA	32
C. parapsilosis 5	Blood	AB861488	FLC, ITRA	128
C. parapsilosis 6	Blood	AB861486	FLC, ITRA, VRZ	80,6
C. parapsilosis 7	Blood	AB861487	FLC, VRZ	101,6
*C.tropicalis 1***	Blood	AB861490	FLC, ITRA, VRZ	64
C.tropicalis 2	Blood	KJ740181	FLC, ITRA, VRZ	64
C.tropicalis 3	Urine	AB861491	FLC	64
C.tropicalis 4	Blood	KJ740185	FLC, ITRA, VRZ	64
C.tropicalis 5	Blood	AB861493	FLC, VRZ	50,8
C.tropicalis 6	Blood	KF616840	FLC, ITRA	64
C. glabrata 1	Blood	AB861484	FLC, ITRA, VRZ	64
C.parapsilosis ATCC 22019	-	-	-	32
C.krusei ATCC 6258	-	-	-	64

[a] Yeasts isolated from biological samples. FLC: Fluconazole; ITC: Itraconazole; VRZ: Voriconazole
[b] MIC_{50} value was defined as the lowest concentration that produced a 50% reduction in the growth of fungal cells after 24 h incubation.
*Strains used to investigate the mechanism of action of palmatine. ** Strains used for the biofilm assay.

An analysis of DNA damage was performed using comet assay, alkaline version, which can detect single and double DNA strand breaks. Double strand lesions are difficult to repair, and may result in cell death or in deleterious mutations. Furthermore, simple breaks can be converted to double breaks during the replication process (Da Silva *et al.,* 2014). Palmatine induced an intense DNA damage in *C. albicans* cells. Some studies demonstrate that palmatine binds strongly to DNA (Bhadra, Maiti, Kumar, 2007; Mi *et al.,* 2015). In order to verify whether palmatine causes oxidative damage to DNA, the comet assay, alkaline version, was also performed in the presence of the FPG enzyme. This enzyme improves the specificity of the test by identifying oxidized purine bases (adenine and guanine).

In this study, there was no difference between the comet assay in the absence and the presence of the FPG enzyme, showing that palmatine does not cause oxidation of purine bases. Similar data were obtained by Da Silva *et al.,* (2016) who showed that berberine induces severe damage to DNA, but that oxidative damage does not occur since results are similar in the presence and absence of the FPG enzyme.

During apoptosis, phosphatidylserine (PS), an inner membrane phospholipid, is externalized to the surface of the cell membrane, signaling that the cell is phagocytized by macrophages. Annexin V is a marker that has a high affinity for PS and binds to the PS externalized in cell undergoing apoptosis (Hwang *et al.,* 2012; Choi and Lee, 2015). Palmatine, at the three concentrations, produced an increase in phosphatidylserine externalization in *C. albicans* cells, indicating that these cells are in an early apoptosis process. Similar data were also verified by (Da Silva *et al.,* 2016) upon performing the same test using berberine in *C. albicans*.

Many studies show that palmatine binds strongly to DNA and attempt to elucidate how this process occurs (Bhadra, Maiti, Kumar, 2007; Mi *et al.,* 2015; Dumont and Monari, 2015). According to the results of this study, it can be proposed that palmatine acts at the DNA level causing secondary changes that, by secondary processes, cause mitochondrial depolarization, leading to fungal cell death by apoptosis, as demonstrated by the externalization of phosphatidylserine test.

Palmatine showed an activity in *Candida* spp. resistant to azoles in planktonic cells and also in the form of biofilms. When microorganisms are organized in the form of biofilms, they have a rather higher resistance to antifungal agents when compared to their planktonic form (Bonhomme and d'Enfert, 2013). Two methodologies were used to verify the antifungal action in biofilms. The first considers the biofilm already formed; it is assessed whether the drug is able to eradicate an existing biofilm. The other methodology is inhibition of the formation of biofilm, i.e., the drug must act preventing the formation of biofilm generally by disrupting cell adhesion processes (Rane *et al.,* 2014; Sun, Liao, Wang, 2015).

The initial stage of biofilm formation is adhesion to a surface influenced by abiotic factors such as hydrophobicity and biotic factors such as increased production of adhesin and other surface proteins. Therefore, the inhibition of adhesion of *Candida* species is a promising target to halt the early stages of biofilm formation. The results of this study show that palmatine acts both in formed biofilms and biofilms in formation in the three tested species of *Candida* resistant to azoles. This is a key characteristic for a drug with a suitable antifungal action, since an ideal antifungal drug should have both the ability to kill the biofilm and to inhibit its formation (Theberge *et al.,* 2013).

Valentin *et al.,* (2012) shows that azoles, including voriconazole, have no activity against pre-formed *Candida* spp. biofilm. This resistance may be explained by the high expression of efflux pumps at the early stages of biofilm formation and low levels of ergosterol at the intermediate and late stages. However, fluconazole and caspofungin may prevent the formation of biofilm when the surface over which it is formed is pretreated with such drugs or when the biofilm is formed in the presence of these drugs. In the 24-hour formed biofilm, palmatine could decrease by 50% the viability of the biofilm in *C. tropicalis* strains resistant to azole at the concentration 10x MIC. The same test performed by (Da Silva *et al.,* 2016) showed that berberine was able to produce the same inhibition rate at the concentration 4x MIC for a strain of *C. tropicalis* resistant to fluconazole. These results are very promising since biofilms are difficult to eradicate, representing continuous infection outbreaks (Valentin *et al.,* 2012; Yu *et al.,* 2008).

An evaluation of viability cell in *C. albicans* resistant to azoles after 3, 6, 12 and 24 h of treatment with palmatine at the MIC, 2x MIC and 4x MIC. * $P < 0.05$ compared to the results for the control, determined by ANOVA followed by the Newman-Keuls test. An evaluation of ROS formation in *C. albicans* resistant to azoles after 3, 6, 12 and 24 h of treatment with palmatine at the MIC, 2x MIC and 4x MIC. * $P < 0.05$ compared to the results for the control, determined by ANOVA followed by the Newman-Keuls test.

Effects of different treatments on the mitochondrial transmembrane potential in strain of *C. albicans* resistant to azoles exposed for 3, 6, 12 and 24 h to palmatine (MIC, 2x MIC and 4x MIC) and Ampho (4 µg/mL). * $P < 0.05$ compared to the results for the control, determined by ANOVA followed by the Newman-Keuls test.

Effect of palmatine at the MIC, 2x MIC and 4x MIC on phosphatidylserine externalization in *C. albicans* resistant to azoles for 3, 6, 12 and 24 h of treatment. * $P < 0.05$ compared to the results for the control, determined by ANOVA followed by the Newman-Keuls test.

Effects of different treatments on the DNA damage index and on the distribution of oxidative DNA damage in *C. albicans* resistant to azoles determined using of the comet assay. *C. albicans* was exposed to RPMI (control), palmatine (MIC, 2x MIC and 4x MIC) and amphotericin B (4 µg/ml). * $P < 0.05$ compared to the results for the control, determined by ANOVA followed by the Newman-Keuls test.

Effect of different concentrations of palmatine on the metabolic activity of biofilms formed of *C. albicans* resistant to azoles analyzed by the MTT reduction assay. * $P < 0.05$ compared to the results for the control, determined by ANOVA followed by the Tukey test.

Effect of different concentrations of palmatine on inhibition of biofilm formation in *C. albicans* resistant to azoles analyzed by the MTT reduction assay. * $P < 0.05$ compared to the results for the control, determined by ANOVA followed by the Tukey test.

Palmatine has an antifungal activity against *Candida* strains resistant to azoles. Its mechanism of action involves mitochondrial depolarization and DNA damage, leading to cell death by apoptosis. Palmatine was able to reduce the viability of the formed biofilm and to inhibit the formation of biofilms in *Candida* spp. resistant to azoles.

Because there is no cytotoxicity regarding mammalian cells, palmatine can be considered a potential candidate for a new drug with an anti-candida action.

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